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Identifying Targets to Prevent Aminoglycoside Ototoxicity

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Abstract

Aminoglycosides are potent antibiotics that are commonly prescribed worldwide. Their use carries significant risks of ototoxicity by directly causing inner ear hair cell degeneration. Despite their ototoxic side effects, there are currently no approved antidotes. Here we review recent advances in our understanding of aminoglycoside ototoxicity, mechanisms of drug transport, and promising sites for intervention to prevent ototoxicity.

Introduction

Ototoxicity encompasses damage to the inner ear auditory (cochleotoxic) and/or vestibular organs (vestibulotoxic). Drug ototoxicity is defined as reversible or irreversible damage to the inner ear that results in loss of function. Although it is preferable to avoid using ototoxic drugs, their benefits often outweigh the risks. The most common ototoxic drugs include aminoglycoside (AG) antibiotics, platinum-based chemotherapeutic drugs, certain antimalarials, loop diuretics, and NSAIDs. Table 1 lists some of the common drugs from the above categories that are known to be ototoxic.

This review focuses on the clinical importance of AGs and their side effects with a major focus on their impact in the cochlea. This review delves into existing model systems for AG research and their translational relevance, mechanisms by which the AG could enter the cochlea and the hair cells (HCs), and the potential mechanisms that could trigger HC death. Each topic is explored from the perspective of a target site for intervention. We refer the avid readers to several other recent excellent reviews on cisplatin ototoxicity (Prayuenyong et al., 2021; Steyger, 2021).

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Aminoglycosides

AG-aminocyclitol antibiotics, commonly known as AGs are water-soluble, cationic molecules that exhibit broad-spectrum antibacterial properties. They are primarily used in the treatment of infections caused by both gram-positive and gram-negative bacteria, mycobacteria, and sometimes by amoeboid and protozoa (Berman and Fleckenstein, 1991). Due to their poor absorption through oral administration, they are generally administered intravenously (Edson and Terrell, 1991).

The first AG to be discovered was streptomycin (Schatz et al., 1944) followed by neomycin (Waksman and Lechevalier, 1949), which formed a base for the discovery and characterization of other similar clinically important AGs as shown in Table 2. AGs are either natural (produced by the bacteria species actinomycetes) or semisynthetic derivatives of natural AGs. For example, amikacin is a semisynthetic derivative of kanamycin B (Table 1). There are no known AGs in clinical use that are derived entirely from chemical synthesis.

Types and structure of aminoglycosides

AG antibiotics can be broadly divided based on the origin as natural and semi-synthetic (Table 2). They can also be divided structurally based on their six-membered aminocyclitol rings. Based on their structure, AGs fall into two groups, those that incorporate a 2-deoxystreptamine ring and those that do not (Table 3). The first group is further subdivided into compounds that are substituted at either the 4 and 5 positions or 4 and 6 positions of the 2-deoxystreptamine ring (Table 3).

General use of aminoglycosides

AGs have a wide range of clinical uses. They are used to treat gram-negative and -positive bacterial infections. Streptomycin is still occasionally used in the treatment of tuberculosis despite the availability of other less ototoxic first line drug regimens (Armstrong et al., 1950; Musser, 1995). AGs block the production of HIV-1 by binding the viral regulatory protein Rev. (Wang et al., 1997; Zapp et al., 1993). AGs are frequently used in patients suffering from cystic fibrosis. AGs can effectively penetrate the sputum allowing them to effectively fight *Pseudomonas aeruginosa*. For example, tobramycin was found to display synergism with β-lactam antibiotics like ceftazidime (den Hollander et al., 1997). AGs are the antibiotics of choice for complicated urinary tract infections (Goodlet et al., 2018), peripartum and neonatal infections (Kankuri et al., 2003; Kent et al., 2014).

General mechanism of AG bacterial entry and action (Fig. 1)

Positively charged AG molecules promote bacterial uptake by binding to the negatively charged components of the bacterial membrane, such as the phospholipids and teichoic acids of gram-positive bacteria and the phospholipids and lipopolysaccharide of gram-negative bacteria (Davis, 1987; Hancock et al., 1991; Taber et al., 1987). Stabilized cross-links between adjacent lipid components in the outer membrane are disrupted by competitively displacing bridging divalent cations (Hancock et al., 1981; Peterson et al., 1985). The disruption induces membrane blebbing, leading to transient holes in the bacterial cell

envelope (Martin and Beveridge, 1986). The holes enhance the permeability of AGs into the bacteria (Kadurugamuwa et al., 1993). Moreover, porins which are transmembrane pore-forming proteins in outer membranes, are also considered as an entry site of AGs (Nakae and Nakae, 1982). The initial entry of AGs into the periplasmic space of the bacteria occurs in a passive and energy-independent manner (EDP-I) (Bryan and Van Den Elzen, 1977). On the other hand, the ensuing steps, including entry into the cytoplasm through the inner membrane, occur in an energy-dependent manner (EDP-II) (Kislak, 1972; Martin et al., 1972; Taber et al., 1987). Various mutations in bacterial genes which are involved in energy metabolism lead to AG resistance. Once AGs access the cytoplasm, they irreversibly bind to the decoding site at the 16S rRNA in the 30S subunit of the ribosome (Garvin et al., 1974). The binding disturbs the recognition and selection of tRNA, leading to misreading during translation (Davies and Davis, 1968a). More specifically, AGs targeting translation interfere with either the assembly or the processing of the ribosome, or with the proper utilization of tRNAs and translation factors (Cabanas et al., 1978; Fredrick and Noller, 2003; Kavcic et al., 2020; Misumi et al., 1978; Wang et al., 2012). Ultimately, these perturbations either produce mistranslated proteins or inhibit protein synthesis. Furthermore, subsequent AG entry into the bacteria is facilitated by the mistranslated proteins (Davis, 1987; Davis et al., 1986; Nichols and Young, 1985). The additional uptake accelerates death of the bacteria. Interestingly, each AG has a different binding affinity for individual rRNA binding sites (Feldman et al., 2010; Fourmy et al., 1998; O'Sullivan et al., 2018; Wasserman et al., 2015; Ying et al., 2019). For antibacterial activity, AG-induced reduction in nucleotide mobility at rRNA is a more important determinant than the binding affinity (Kaul et al., 2006). These differences between multiple types of AGs are considerable for antibacterial potency and bacterial resistance.

Aminoglycoside nephro- and ototoxicity

The side effects of AGs have been established for many years (Hinshaw et al., 1947). AGs cause nephrotoxicity with repeated usage. In renal tubular cells, AGs can enter through endocytosis-dependent and independent manner (Nagai and Takano, 2014). AGs accumulate in lysosomes, leading to cell necrosis. Continued exposure can cause renal failure (Mingeot-Leclercq and Tulkens, 1999; Swan, 1997). AGs are known to enter both outer hair cells (OHCs) and inner hair cells (IHCs) with OHCs being more sensitive to AG damage (Forge and Schacht, 2000). Continued accumulation of AG antibiotics in HCs lead to their demise along the basal to apical axis (Cheng et al., 2003; Cunningham et al., 2002; Fausti et al., 1984; Forge and Schacht, 2000; Matsui et al., 2004). AG ototoxicity usually manifests itself as high frequency hearing loss consistent with an increasing apical-to-basal gradient of damage; prolonged usage extends damage to lower frequency range and apical HC loss (Zettner and Gleser, 2018).

Animal models for studying ototoxicity (Table 4)

To study mechanisms underlying AG-induced ototoxicity, various animal models have been generated with different type, dosage and application method for AGs as well as co-administration of diuretics. Animals such as mice, rats, guinea pigs, gerbils, and chinchilla were used for studying AG-induced ototoxicity. AGs are systemically delivered

via intramuscular, intraperitoneal or subcutaneous injections. Most studies apply multiple lower doses of AGs on successive days rather than a single, high dose injection to induce ototoxicity. This multi-dose injection strategy can avoid animal mortality sometimes resulting from a single, high dose injection (Murillo-Cuesta et al., 2010; Taylor et al., 2008; Wu et al., 2001). Examples of animal models for the irreversible ototoxicity induced by AGs are summarized in Table 4. Doses in all of these models are typically much higher than used clinically but are needed in order to consistently generate damage.

Diuretics are generally administered simultaneously with AGs to induce robust sensory HC loss (McFadden et al., 2002). In particular, mice often need co-administration of diuretics to induce ototoxicity as they are highly resistant to AGs, presumably because of high excretion rates (Blakley et al., 2008; Murillo-Cuesta et al., 2010; Ogier et al., 2020; Taylor et al., 2008; Wu et al., 2001). Experimental studies showed the co-administration of diuretics with AGs potentiate AG-induced ototoxicity. However, how diuretics affect AG toxicity is not clear. Here are several possible mechanisms. Some studies reveal that diuretics inhibit strial enzymes Na^+, K^+-ATPase and adenylate cyclase (Kusakari et al., 1978; Paloheimo and Thalman, 1977). The enzyme inhibition induces swelling of the stria vascularis, leading to rapid decrease of the endocochlear potential as well as eventual loss of the cochlear microphonic, summating, and compound action potentials (Kusakari et al., 1978; Rybak et al., 1991). Due to the attenuation of the endocochlear potential, administration of diuretics induces temporary hearing loss (Ding et al., 2016). Even though swelling of the stria vascularis typically fully recovered within a day (Ding et al., 2016), simultaneous administration of diuretics and AGs can potentiate ototoxicity (Ding et al., 2010). It is possible that diuretics damage the tight cell junctions in the stria vascularis and disrupt the blood-labyrinth barrier (BLB), further increasing AG uptake into HCs (Ding et al., 2016; Liu et al., 2011). Another possible mechanism is that osmotic swelling caused by diuretic-induced inhibition of Na-K-2Cl cotransporter activates TRPV4 channel in stria vascularis (Jiang et al., 2017a). More AGs can enter the cochlea through the activated TRPV4 channels, resulting in enhanced ototoxicity. The increased endolymph AG would then promote access through MET channels (see uptake mechanisms in later sections) despite the driving force for current through the MET channel being reduced by the loss of the endocochlear potential. Another possibility is that the diuretic reduces filtration within the kidney so that AG blood levels remain elevated for longer, resulting in more time for entry into the cochlea. And finally, as diuretics can be ototoxic on their own (Table 1), it is possible that the diuretic effects are simply summing with the AGs effect to cause greater toxicity. Thus, existing models that require diuretics may be flaweds because we do not truly understand the mechanism by which the synergy is happening; however, they remain the available models of choice for small animal testing.

Mechanism of AGs transport into the cochlea (Fig. 2)

The cochlea has two main fluid compartments, endolymph and perilymph, which are divided into three chambers: the scala vestibuli (SV), scala media (SM), and scala tympani (ST) (Pickles, 2008). The endolymph has a unique ionic composition, high K^+ and low Ca^{2+}, while the perilymph has a similar ionic composition to extracellular fluid (Sterkers et al., 1984, 1988). The stria vascularis maintains the ionic environments which are critical

for cochlear function (Wangemann, 2006). In the stria vascularis, dense capillaries and transporters selectively regulate endolymph composition (Wangemann, 2006). Furthermore, BLB, which is composed of tight junction-coupled endothelial cells, controls exchange between blood and intracochlear fluids so that entry of macromolecules into the cochlea is tightly regulated (Nyberg et al., 2019). The organ of Corti (OoC) is a specialized sensory structure that sits on the basilar membrane within the cochlea, containing IHCs and OHCs as well as at least seven types of supporting cells (Pickles, 2008). The hair bundle, comprised of actin filled stereocilia is an essential HC organelle critical for the conversion of mechanical stimulation into electrical signals (Pickles, 2008). The hair bundle sits atop the HC and faces the endolymph solution. Mechanotransducer (MET) channels at the tips of stereocilia open upon deflection of stereocilia and allow HC activation (Pickles, 2008). Furthermore, transient receptor potential (TRP) and adenosine triphosphate (ATP) receptor channels (Fettiplace, 2017; Housley et al., 1992; Housley et al., 1999) are also found on the stereocilia.

The first step in understanding mechanisms of AG-induced ototoxicity is to determine how AGs enter the cochlea and their cells. To track the AG entry route, various experimental strategies such as *in vitro* assays, immunolabeling and AG-conjugated to fluorescent labels have been used in neonatal or adult cochlear tissues (Alharazneh et al., 2011; Dai et al., 2006; Imamura and Adams, 2003; Kawashima et al., 2011; Marcotti et al., 2005; Wang and Steyger, 2009). Gentamicin Texas Red (GTTR) is the most often used fluorescent-conjugated AG and a powerful tool to study AG trafficking into the cochlea (Dai et al., 2006; Wang and Steyger, 2009). One caveat to GTTR is that it is a mixture of gentamicin subtypes and the labeled amine is not well targeted, so care must be taken in interpretation of results. Here, we review the findings on mechanisms of AGs entry into the cochlea and as well as their actions therein.

Mechanism and therapeutic target 1: AG access into the cochlea (Fig. 3)

Systemically administrated fluorescent-tagged AGs are first detected in the capillaries in stria vascularis in mice and guinea pigs (Dai et al., 2006; Imamura and Adams, 2003; Wang and Steyger, 2009) ([1] in Fig. 3). The GTTR is further detected in cell subtypes within the stria vascularis (marginal, intermediate and basal cells), suggesting drug transport across this region (Wang and Steyger, 2009) ([2] in Fig. 3). BLB is one of the initial sites of AG entry from the blood into the interstitial space in the cochlea (Dai et al., 2006; Hawkins, 1973). Multiple factors such as active and passive transports, ion channels, blood flow, inflammation, free radicals and noise exposure can influence the permeability of the BLB, possibly potentiating AG cochlear uptake (Shi, 2016). For example, many studies reveal that inflammation enhances AG uptake into the cochlea (Chai et al., 2021; Gu et al., 2021; Hirose and Li, 2019; Jiang et al., 2017b; Koo et al., 2015). *In vivo* work showed that AGs transport into endolymph across the strial BLB (Li and Steyger, 2011) ([3] in Fig. 3). Endolymph is considered as a predominant route for AG uptake in HCs (also see next section) compared to the uptake from the perilymph (Li and Steyger, 2011). On the other hand, one early study showed that the AG level in perilymph is higher than that in endolymph, suggesting AG uptake into the perilymph occurs (Tran Ba Huy et al., 1986) ([4] in Fig. 3). Reissner's and basilar membranes are further considered as possible entry

routes of AG from the perilymph to the endolymph (Huy et al., 1983; Tran Ba Huy et al., 1986; Tran Ba Huy et al., 1981) ([5] in Fig. 3). Furthermore, the osseous spiral lamina maybe a potential AG entry route because increasing matrix metalloproteinases can enhance the permeability to AGs between the ST and spiral ganglion neurons (Li et al., 2017). After systemic application, AGs are detected in multiple locations in the cochlea such as HCs, Deiters' cells, interdental cells, mesenchymal cells, fibrocytes, nerve fibers, and spiral ganglion neurons (Dai et al., 2006; Imamura and Adams, 2003) (orange circles in Fig. 3). The AG uptake into multiple cochlear cells are shown based on either localization of GTTR (Dai et al., 2006; Wang and Steyger, 2009) or AG immunolabeling (Imamura and Adams, 2003). Both of these approaches relied on fixed tissues (Imamura and Adams, 2003; Wang and Steyger, 2009). Live cell imaging shows AG uptake selectively into HCs and perhaps spiral ganglia fibers (Alharazneh et al., 2011), not supporting cells. Similarly, HCs die when treated with AGs, not supporting cells. Thus, the discrepancy between fixed and live cell data needs to be reconciled in future studies. There is strong agreement that HCs uptake AGs regardless of technologies used for assessment (Dai et al., 2006; Imamura and Adams, 2003). Despite much effort, it remains unclear as to how AGs enter the endolymph from stria vascularis and target multiple cell types, with transporters, ion channels, and transcytosis serving as possible mechanisms. As described in Figure 3, the stria vascularis is considered as a first site where AGs are taken up from the bloodstream to the cochlea (Dai et al., 2006; Imamura and Adams, 2003; Wang and Steyger, 2009). BLB borders the stria vascularis and intracochlear fluid and can be a major target for blocking AG entry (Dai et al., 2006; Hawkins, 1973). As we mentioned above, the permeability of the BLB can be modulated by multiple factors such as diuretics, active and passive membrane functions, ion channels, blood flow, inflammation, free radicals and noise exposure (Chai et al., 2021; Ding et al., 2016; Hirose and Li, 2019; Koo et al., 2015; Liu et al., 2011; Shi, 2016). Numerous transporters and ion channels in the stria vascularis are potential therapeutic targets to block AG uptake (Patuzzi, 2011). Very recently, *in vivo* real-time observation revealed that AG trafficking into the cochlea is mediated by an endocytic receptor megalin (Kim and Ricci, 2022) which is expressed in the stria vascularis, tightly binds AGs and is required for AG transport in the kidney (Christensen and Birn, 2002; Dagil et al., 2013; Hori et al., 2017; Tauris et al., 2009). Numerous ligands, such as megalin, serve as candidate competitive inhibitors of AGs, possibly blocking AG transport into the cochlea (Christensen and Birn, 2002; Dagil et al., 2013; Hori et al., 2017; Tauris et al., 2009). Another possible target for initial AG entry route into cochlea are TRP channels such as TRPV1, TRPV4, and TRPC3, which are expressed in stria vascularis (Jiang et al., 2019; Karasawa et al., 2008; Phan et al., 2010). However, at present, the exact mechanisms governing BLB permeability are largely unknown, a knowledge gap that beckons investigation leading to discovery of novel therapeutic options for preventing AG trafficking into intracochlear spaces.

Mechanism and therapeutic target 2: AG access into cochlear hair cells (Fig. 4)

Once AGs enter the endolymph, OHCs and IHCs in the OoC uptake AGs (Dai et al., 2006; Imamura and Adams, 2003). Previous studies showed that not only multiple channels but

also cellular process are involved in AG transport into HCs. Here, we will discuss MET, TRP, and ATP channels as well as endocytosis as entry routes of AG uptake in HCs.

The MET channel is a non-selective cation channel located at the tips of the HC stereocilia. The MET channel is unique to sensory HCs and has pore dimensions estimated to be large enough for AG permeation (Farris et al., 2004; Marcotti et al., 2005). A number of studies point to the critical role for MET channels in AG entry into HCs (Alharazneh et al., 2011; Kawashima et al., 2011; Majumder et al., 2017; Makabe et al., 2020; Marcotti et al., 2016; Marcotti et al., 2005; Ricci, 2002; Vu et al., 2013). The polycationic AGs is presumed to approach the negatively charged channel pore for HC entry. In cochlear explants, MET channel blockers such as amiloride, quinine or curare suppressed a rapid transport of AGs into HCs, leading to prevention of HC loss (Alharazneh et al., 2011; Kawashima et al., 2011; Majumder et al., 2017; Marcotti et al., 2016; Marcotti et al., 2005; Vu et al., 2013). Furthermore, high extracellular calcium levels reduce the open probability of the MET channels, which in turn reduces AGs ability to enter through the MET channels resulting in diminished ototoxicity (Coffin et al., 2009; Ou et al., 2012; Vu et al., 2013; Wang and Steyger, 2009). Reduced AG uptake and toxicity are also observed in several genetic models where MET channels are rendered non-functional such as mutants of *Cadherin23*, *Myosin7a*, and *Transmembrane channel-like proteins* (*TMC*) (Kawashima et al., 2011; Marcotti et al., 2016; Vu et al., 2013; Wang and Steyger, 2009). Taken together, MET channels are the main mediator of AG entry into the HCs. Once inside HCs, a high energy barrier in the entrance does not allow AGs to exit from the cytosol via MET channels (Farris et al., 2004; Marcotti et al., 2005; Pan et al., 2012; van Netten and Kros, 2007), thus leading to drug accumulation within the cell.

In addition to the MET channels, TRP channels may also mediate AG entry into HCs. The TRP channel is also a non-selective cation channel that is permeable to Ca^{2+} ions (Karasawa et al., 2008; Myrdal and Steyger, 2005; Nilius and Szallasi, 2014). The pore sizes of TRPA1 (~1.1–1.4 nm) (Karashima et al., 2010), TRPV1 (~1 nm) (Jara-Oseguera et al., 2008) and TRPC3 (~6 nm) (Mio et al., 2007) are also considered to be large enough to allow AGs entry into HCs. Several subtypes of TRP channel such as TRPA1, TRPV1, TRPV4 and TRPC3 are expressed in the cochlea (Asai et al., 2010; Cuajungco et al., 2007; Garcia-Anoveros and Duggan, 2007), and candidates for AG permeation into HCs (Karasawa et al., 2008; Myrdal and Steyger, 2005; Stepanyan et al., 2011; Zheng et al., 2003). For example, TRPA1 channels activated by lipid peroxidation products allow AG entry when the MET channel is disabled (Stepanyan et al., 2011). Noise exposure also activates TRPA1 channels, leading to an increase of AG entry into HCs (Li et al., 2015; Yamashita et al., 2004). Furthermore, TRPV1 and TRPV4 are also considered as AG-permeant channels (Karasawa et al., 2008; Myrdal and Steyger, 2005; Zheng et al., 2003). More specifically, inhibitors of TRPV1 and TRPV4 channels markedly reduce AG uptake into HCs, though these compounds also can block MET channels (Lee et al., 2013). Inflammation-induced expression of TRPV1 possibly at multiple locations lead to an overall increased in cochlear uptake of AGs (Jiang et al., 2019). The main issue with TRP channels as a primary target for AG entry is that under normal physiological conditions these channels are typically not open. However, under conditions of infections or if initial access is via MET channels and that pathology activates TRP channels, it could result in a potentiation of the toxicity response.

Some studies suggest that AGs enter HCs through ATP receptor mediated ion channels (Bongartz et al., 2010; Lin et al., 2019; Xi et al., 1993). For example, prolonged activation of ATP receptors aggravates AG-induced ototoxicity in IHCs and OHCs (Lin et al., 2019). In Xenopus laevis oocytes, AGs inhibit ATP-induced receptor currents (Bongartz et al., 2010). Moreover, AGs inhibit the ATP responses in OHCs, suggesting ATP receptors as candidate targets of AGs (Xi et al., 1993).

Endocytosis at the apical and synaptic poles of HCs is another potential route of AG entry into HCs (Hashino et al., 2000). AGs are found in membrane-bound vesicles in the HCs, and the AG-contained vesicles increase over time (Hashino et al., 2000). In the zebrafish lateral line HCs, live imaging revealed endocytic uptake of AGs (Hailey et al., 2017; Nagai and Takano, 2014). The uptake results in accumulation of AGs in lysosomes (Hailey et al., 2017; Hashino et al., 2000; Nagai and Takano, 2014). In addition to its effects on the MET channel, Myosin VIIa may be involved in endocytosis-mediated AG accumulation evidenced by its high expression at the apical end of HCs where high amounts of vesicles are found (Richardson et al., 1997). By contrast, some studies suggested that inhibition of endocytosis does not affect AG uptake into HCs (Alharazneh et al., 2011; Myrdal et al., 2005). Multiple forms of endocytosis exist and assurance of pharmacological antagonism of all pathways is difficult, possibly causing the conflicting results.

As we reviewed above, MET, TRP, ATP channels and endocytosis are proposed as the entry routes of AGs into HCs. In particular, the MET channel is a major entry route for AGs (Alharazneh et al., 2011; Kawashima et al., 2011; Majumder et al., 2017; Marcotti et al., 2016; Marcotti et al., 2005; Vu et al., 2013), as multiple types of MET channel blockers have been found to protect against AG-induced HC loss (Alharazneh et al., 2011; Kawashima et al., 2011; Marcotti et al., 2016; Marcotti et al., 2005; Vu et al., 2013). In spite of an otoprotective effect of the blocker against HC loss *in vitro*, some of these blockers may not be suitable for clinical use as they are rather toxic. Targeting other ion channels or endocytosis is challenging, as these pathways are neither specific nor predominant to HCs. In recent years, many analogs of MET channel blockers have been identified as promising otoprotective agents against AGs. For example, d-tubocurarine was identified as a rapid and reversible blocker of MET channel and was used to reduce AG uptake into HCs (Farris et al., 2004; Glowatzki et al., 1997; Kirkwood et al., 2017). Although the blocker induces a transient loss of hearing and remodeling of the stereocilia, those changes are reversible (Velez-Ortega et al., 2017). Berbamine is also a reversible blocker and is itself highly toxic to HCs at high concentrations; however, berbamine analogs were less toxic and maintained otoprotective effects (Hudson et al., 2020; Kirkwood et al., 2017). Another reversible MET channel blocker, phenoxybenzamine, confers significant protection to HCs, but shows ototoxicity at higher concentrations and an additive toxic effect when combined with neomycin (Majumder et al., 2017). Furthermore, large-scale screening identified several protectants such as ORC-13661 and UoS-7692, both of which reversibly block MET channels and confer otoprotection (Kenyon et al., 2021; Kenyon et al., 2017; Kitcher et al., 2019). The otoprotective effects of UoS-7692 has been verified in zebrafish *in vivo* and cochlear explants *in vitro* (Hudson et al., 2020; Kenyon et al., 2017; Kirkwood et al., 2017; Kitcher et al., 2019; Majumder et al., 2017). Furthermore, ORC-13661 has been validated to be protective *in vivo* in the rat cochlea. Thus, MET channel blockers are

promising otoprotectants for AG-induced hearing loss in clinic. However, more studies on the potential effects of MET channel blockage on hearing function are required to determine the safety of these drugs.

Mechanism and therapeutic target 3: Intracellular mechanism (Fig. 5)

In the inner ear, after AG treatment, OHCs express more apoptotic markers than IHCs, with HCs in the basal turn showing more damage than those in the apical turn (Cheng et al., 2003; Cunningham et al., 2002; Forge and Li, 2000; Matsui et al., 2004). A large body of work has shown that AGs affect ribosomes, protein translation, ion transport, and lipids. (Forge and Schacht, 2000).

Ribotoxicity

Major clinically used antibiotics like AGs, tetracyclines, and macrolides target ribosomes thereby disrupting protein synthesis. AGs are antibiotics that specifically bind to the ribosomal RNA and cause misreading of the RNA code (Borovinskaya et al., 2007; Davies and Davis, 1968b; Feldman et al., 2010). RNA regions of the ribosomes are important for their translational activity and are conserved among organisms (Noller, 1991). Though chemically distinct, AGs bind to the conserved A-site of the 30s ribosomal subunit. This binding of AG stabilizes mRNA-tRNA binding and thereby decreases the tRNA dissociation leading to proofreading errors (Karimi and Ehrenberg, 1994). In eukaryotic cells, AG is shown to affect mitochondrial translation, which has been postulated to mediate its ototoxic side effects in humans (Böttger et al., 2001; Hobbie et al., 2008). In support of this concept, patients with mitochondrial rRNA mutations are highly sensitive to AGs. The mutations in the mitochondrial small ribosomal RNA genes A1555G and C1494T map to aminoacyl-tRNA A-site in a bacterial ribosome, resulting in inhibition of translocation of the mRNA-tRNA complex (Böttger et al., 2001; Campuzano et al., 1979; Carter et al., 2000; Davies and Davis, 1968b; Hutchin and Cortopassi, 1994; Moazed and Noller, 1987; Prezant et al., 1993; Zhao et al., 2004). This specific site is likely why AGs are more toxic to prokaryotic than eukaryotic cells (Böttger et al., 2001; Recht et al., 1999; Sander et al., 1996). A look into the molecular structure of the porcine 55S mitochondrial ribosome also shows a binding site for AGs in the A-site of the 28S subunit (Greber et al., 2015). This interaction of AGs with the mitochondrial protein synthesis may also affect the eukaryotic mitochondria and exacerbate ototoxicity (Hobbie et al., 2008) potentially through upregulation of reactive oxygen species. A recent study showed that the crystal structure of the 80S eukaryotic cytosolic ribosome in complex with multiple AG compounds (paromomycin, gentamicin, geneticin, and TC007) contains multiple AG binding sites within the large and small subunits of the ribosome; As such, AG binding affects the conformation of the pre-translational complex (Prokhorova et al., 2017). Thus, AGs can affect eukaryotic cytosolic ribosomes by multiple mechanisms and decrease their translational efficiency.

Minimizing ribotoxicity is another potential target to reduce ototoxicity. Modifying the structure of AG compounds to mitigate ribotoxicity while maintaining antibiotic effects is an efficient approach to reduce AG ototoxicity. AG antibiotics have been shown to interact with both the mitochondrial and cytosolic ribosomes in eukaryotic cells. Studies have

shown that modified AGs that preferentially target cytosolic as compared to mitochondrial ribosomes displayed lower levels of ototoxicity (Kandasamy et al., 2012; Shulman et al., 2014), suggesting that the mitochondrial ribosomes may be the primary mediator of AG-induced ototoxicity. AGs with lower affinities to mitochondrial ribosomes can also potentially minimize ototoxicity (Matt et al., 2012; Perez-Fernandez et al., 2014). One study found that drug-binding of 4'−6'-acetyl and 4'-O-ether modification of 2-deoxystreptamine AG displayed less affinity to both cytosolic and mitochondrial ribosome drug binding regions and largely maintained antibacterial activity (Perez-Fernandez et al., 2014). This data suggest that exploiting the differences between the microbial and eukaryotic ribosomes is a potentially feasible approach for reducing ototoxicity. Modifications that minimize AG binding to the ribosome without compromising their antimicrobial activity should be an effective way to minimize AG-induced ototoxicity.

Free radicals

Free radicals are molecules with an unpaired electron making them unstable and highly reactive. Free radicals are normally present and participate in cell signaling and cell death pathways (Phaniendra et al., 2015). The free radical concentration in the cell is tightly regulated by an array of native antioxidants. When the antioxidant system becomes overwhelmed, free radicals can impair protein production, causing DNA and lipid damage and eventually leading to cell death. *In vitro* and *in vivo* studies have shown the production of reactive oxygen species (ROS) post AG treatment (Esterberg et al., 2016; Shulman et al., 2014). AG antibiotics have been reported to increase ROS production in avian HCs (Hirose et al., 1997) and the amount of ROS produced can be correlated with mitochondrial calcium uptake leading to HC death. A study on zebrafish lateral line HCs showed that application of the mitochondrial uniporter Ru360 reduced mitochondrial oxidation showing that mitochondrial calcium drives ROS production during AG-induced HC loss (Esterberg et al., 2016). In this study, ROS scavengers targeted the mitochondria and prevented HC death (Esterberg et al., 2016). In sum, AGs' effects on the ribosomes and endoplasmic reticulum can lead to HC loss by ultimately increasing the ROS levels in the cell and triggering the apoptotic pathway.

Many studies have focused on minimizing the effects of ROS to mitigate ROS-induced cellular damage. Antioxidants conferred significant decrease in HC loss against AG-induced cell death. Aspirin was shown in human studies to be protective against gentamicin-induced hearing loss (Sha et al., 2006). Moreover, α-tocopherol was shown to reduce the progression of hearing loss in albino guinea pigs (Fetoni et al., 2003). *In vitro* screening using cell cultures showed compounds like lipoic acid, resveratrol, thiourea, deferoxamine, salicylic acid having a protective effect (Noack et al., 2017; Sha and Schacht, 2000). Corticosteroids such as dexamethasone also were shown to protect HCs by blocking nitric oxide syntheses and blocking free radicals (Himeno et al., 2002; Park et al., 2004). Hence, antioxidants have been shown to be an effective protective agent against AG ototoxicity. On the other hand, depleting glutathione, which is an intracellular free radical scavenger, did not have a significant impact on HC survival, suggesting that the involvement of free radicals in AG-ototoxicity might be more complex (Majumder et al., 2015). Scavenging ROS might possibly slow but not prevent HC death, as AGs interaction with cytosolic and mitochondrial

ribosomes, lipid interactions and decreasing translational efficiency are unlikely to be protected by antioxidant treatments. The efficacy and the duration of antioxidant treatment will be determined by the rate of clearance of AGs from the HCs, as AGs have been detected in the sensory HCs 11 months post treatment (Aran et al., 1993). In summary, the applicability of and duration of treatment with antioxidants as a protectant against AGs need further investigation.

Calcium signaling

Ca^{2+} is a second messenger with broad physiological functions. Tight regulation of calcium is important as they have a multitude of functions from defining the open probability for the MET channels (Ricci et al., 1998) to playing a major role in exocytosis and endocytosis at IHC synapses (Beutner et al., 2001). In zebrafish, AGs first cause a loss of mitochondrial membrane potential in HCs, after which a transient increase in intracellular Ca^{2+} occurs prior to cell death. Chelating or caging this intracellular Ca^{2+} decreases the toxicity of AGs (Esterberg et al., 2013). Furthermore, there is a tight coupling of Ca^{2+} flow between the endoplasmic reticulum (ER) and mitochondria, with a sharp rise in Ca^{2+} in the ER shortly after a similar even in the mitochondria, subsequently leading to increased ROS production, intracellular damage, and cell death (Esterberg et al., 2014) (Esterberg et al., 2016).

Lipid interactions

AGs are known to interact with the plasma membrane lipids to induce membrane blebbing or phosphatidylserine externalization in the apical surface of the HCs (Goodyear et al., 2008; Richardson and Russell, 1991). AGs are also known to interact with phosphatidylinositol–4,5-bisphosphate (PIP2) and phosphatidylinositol l–3,4,5-trisphosphate (PIP3) (Gabev et al., 1989; Schacht, 1976b). PIP2 was also shown to localize in stereocilia and interact either directly or indirectly with ion channels at that location. PIP2 plays a major role in modulating MET channel (Cunningham et al., 2020; Effertz et al., 2017; Hirono et al., 2004). Free PIP2 amount in the stereocilia was shown to affect MET current adaptation and single-channel conductance (Effertz et al., 2017). AG binding to PIP2 can have an indirect effect on MET channel properties and adversely impact auditory function. PIP2 metabolism is linked to KCNQ4 potassium channel, mutations of which cause hereditary hearing loss by inducing OHC loss (Kharkovets et al., 2006; Kharkovets et al., 2000; Kubisch et al., 1999). AG binding to PIP2 may alter the KCNQ4 channels and could adversely affect the viability of the HCs by causing KCNQ4 dysfunction (Li et al., 2005; Suh et al., 2006).

Conclusions and directions for future work

AGs are potent antibiotics used for severe gram-negative bacterial infections, sepsis and tuberculosis (Schacht et al., 2012). Due to their stability, efficacy, and a low incidence of allergic reactions, AGs are among the most commonly used antibiotics despite their irreversible side effect of ototoxicity (Schacht et al., 2012; Van Boeckel et al., 2014). Understanding the mechanisms of AG uptake into the cochlea, their binding kinetics to different cellular components, and the resulting cellular response is required to identify therapeutic targets to prevent ototoxicity. Numerous efforts have revealed that AGs transport

into cochlea through stria vascularis from blood vessels. Once AGs enter the endolymph, the cells in the OoC, mainly HCs, uptake AGs. MET, TRP, and ATP channels as well as endocytosis were suggested as entry routes of AG uptake in HCs. Intracellular AGs modify ribosomes, protein translation, ion transport, and lipids, leading to the cell death. We have highlighted several areas of knowledge gaps that serve as hurdles to our quest to prevent AG ototoxicity.

1) Understanding AG transport into the inner ear in human and animals. Numerous studies for elucidating the mechanisms underlying AG-induced ototoxicity were done in various animal models (e.g. mice, rats, guinea pigs, gerbils, and chinchilla). To induce AG-induced ototoxicity in animals, high and multiple doses of AGs as well as administration of diuretics are needed as most of the animals have higher resistance to AGs than human (Murillo-Cuesta et al., 2010; Taylor et al., 2008; Wu et al., 2001). The use of concurrent of diuretics and AG and high doses of AGs are both contraindicated in the clinical setting, so the clinical relevance of such paradigms needs to be considered despite their effectiveness and reproducibility as model systems. Moreover, while LPS-induced sepsis increases uptake of AGs into the inner ear, most clinical infections do not cause sepsis. How other forms of systemic infection (e.g. pneumonia or complicated urinary track infection) may affect AG uptake and ototoxicity remains elusive. Lastly, transporters responsible for AG entry from blood to the cochlea are currently unclear, and they may serve as potential targets to prevent AG uptake and ototoxicity in the long term.

2) Separation of ototoxicity and antibacterial activity. An ultimate goal of therapy is to eliminate AG-induced ototoxicity without compromising its antimicrobial effects. Understanding the structure-activity relationships of AGs can help direct our efforts in minimizing toxicity while preserving the antibacterial effects (O'Sullivan et al., 2020). Furthermore, multiple modifications of AGs have shown potential to prevent ototoxicity (Huth et al., 2015). Several validated MET channel blockers will be determined whether they are promising otoprotectants in mammalian models and in the clinic (Kenyon et al., 2021; Kitcher et al., 2019). While this co-therapy approach appears promising, it remains to be determined whether a prolonged regimen of MET channel blockers would be needed since certain AG treatment lasts weeks, and whether MET channel blockers can cause hearing loss is unclear.

3) Preventing intracellular action of AGs. Another way to decrease AG-induced ototoxicity is by targeting its intracellular action. Modifying AG structure so that it can preferentially bind to cytosolic ribosomes instead of mitochondrial ribosomes thereby delaying or preventing HC death (Kandasamy et al., 2012; Shulman et al., 2014). Designing the AGs to minimize binding to the eukaryotic ribosomes (Perez-Fernandez et al., 2014) without compromising their antimicrobial activity should be another major focus of research. Although antioxidants have been shown to prevent AG-induced HC death, its efficacy long-term and whether they interfere with antimicrobial activity remain unclear. A combinatorial approach employing modified AGs with antioxidants might prove to be a more effective approach to combat AG-induced HC loss.

In summary, the use of AGs remains vital in clinical settings worldwide. In addition to targeting individual pathways mentioned, it is conceivable to combine therapy preventing AG entry from the bloodstream in addition to blocking MET channels. It is imperative that future studies better define the mechanism of drug entry, intracellular interactions, and mechanism of HC toxicity to better identify targets in order to eliminate its side effects.

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Abbreviation

AG(s)	aminoglycoside(s)
HC(s)	hair cell(s)
OHC(s)	outer hair cell(s)
IHC(s)	inner hair cell(s)
OoC	organ of Corti
SV	scala vestibuli
SM	scala media
ST	scala tympani
GTTR	gentamicin texas red
MET	mechanotransducer
TRP	transient receptor potential
ATP	adenosine triphosphate
BLB	blood-labyrinth barrier

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Highlights

- Aminoglycosides cause irreversible hearing loss by inducing cochlear hair cell death
- Diuretics and sepsis potentiate aminoglycoside uptake into the cochlea and ototoxicity
- Aminoglycoside enter hair cells via mechanotransducer channels
- Blockers of mechanotransducer channels or designer aminoglycosides prevent hair cell degeneration

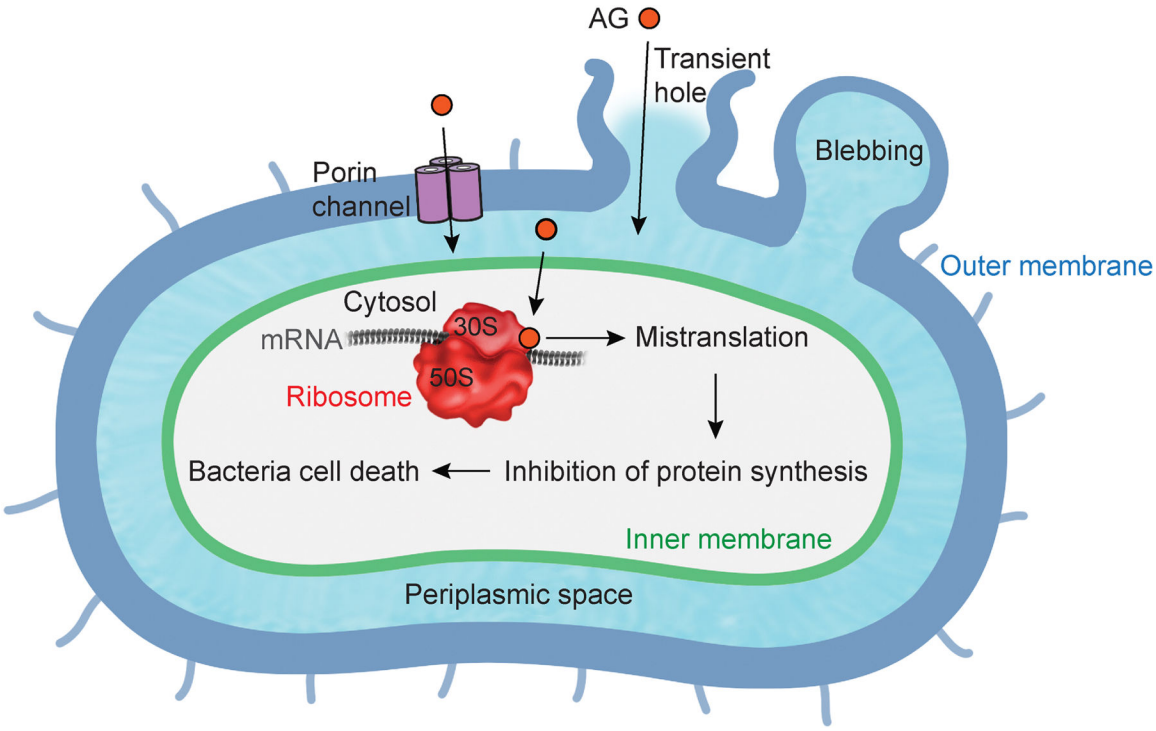

Fig. 1. General mechanisms of aminoglycoside bacteria entry.

Aminoglycoside (AG) enters into a periplasmic space (light blue) through outer membrane (dark blue), and enters into cytosol through inner membrane (green). AG binding to ribosome (red) induces mistranslation, resulting in bacteria cell death.

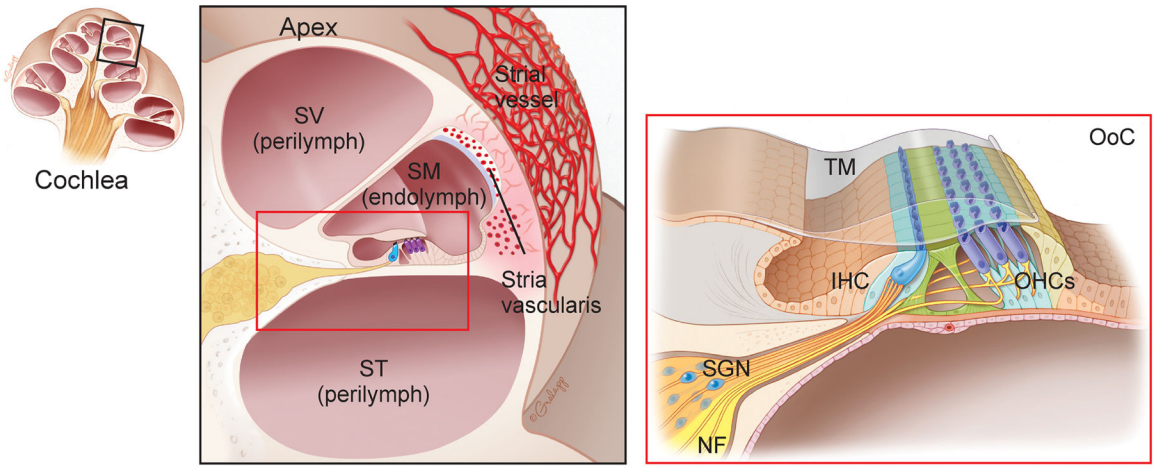

Fig. 2. Anatomy of the mammalian cochlea.

(Left) Cross section of the cochlea. (Center) apical turn of the cochlea (magnified view). Cochlea are composed of three chambers, and filled with endolymph and perilymph. Dense capillaries and transporters in the stria vascularis selectively regulate endolymph composition. SV, scala vestibuli; SM, scala media; ST, scala tympani. (Right) organ of Corti (OoC) (magnified view). OoC contains inner and outer hair cells (IHCs and OHCs) as well as supporting cells. TM, tectorial membrane; SGN, spiral ganglion neuron; NF, nerve fiber.

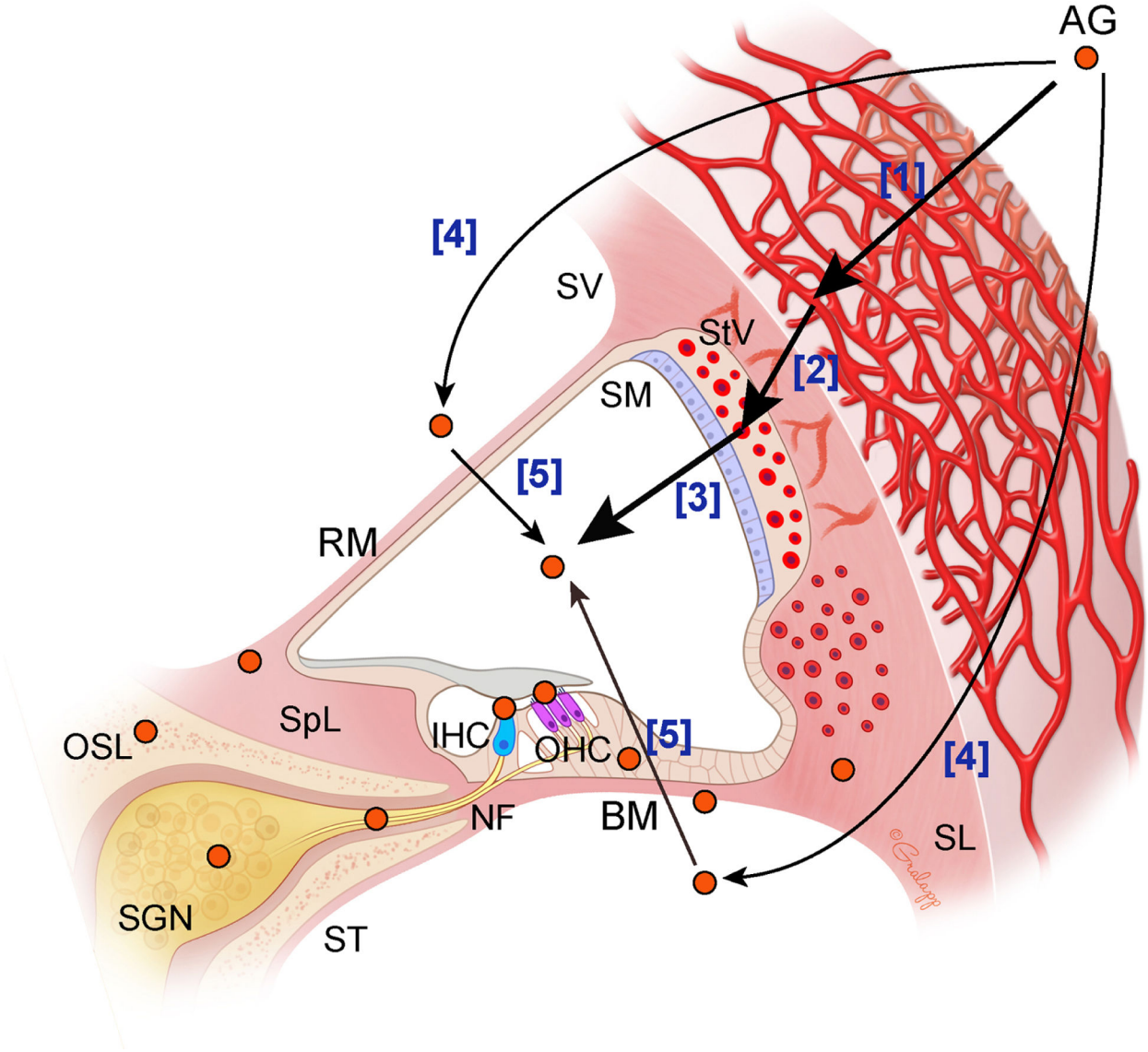

Fig. 3. Proposed mechanisms of aminoglycoside entry into the endolymph.

Aminoglycoside (AG) is detected in the capillaries [1] and individual cell subtypes [2] in the stria vascularis. *In vivo* work showed that AG enters into endolymph [3]. AG uptake into the perilymph has also been suggested [4]. Reissner's and basilar membranes (RM and BM) are also considered as possible entry routes of AG from the perilymph to the endolymph [5]. Thick arrows indicate the AG entry routes with stronger evidences than narrow arrows. SV, scala vestibuli; SM, scala media; ST, scala tympani; StV, stria vascularis; SL, spiral ligament; SpL, spiral limbus; OSL, osseous spital lamina; SGN, spiral ganglion neuron; NF, nerve fiber.

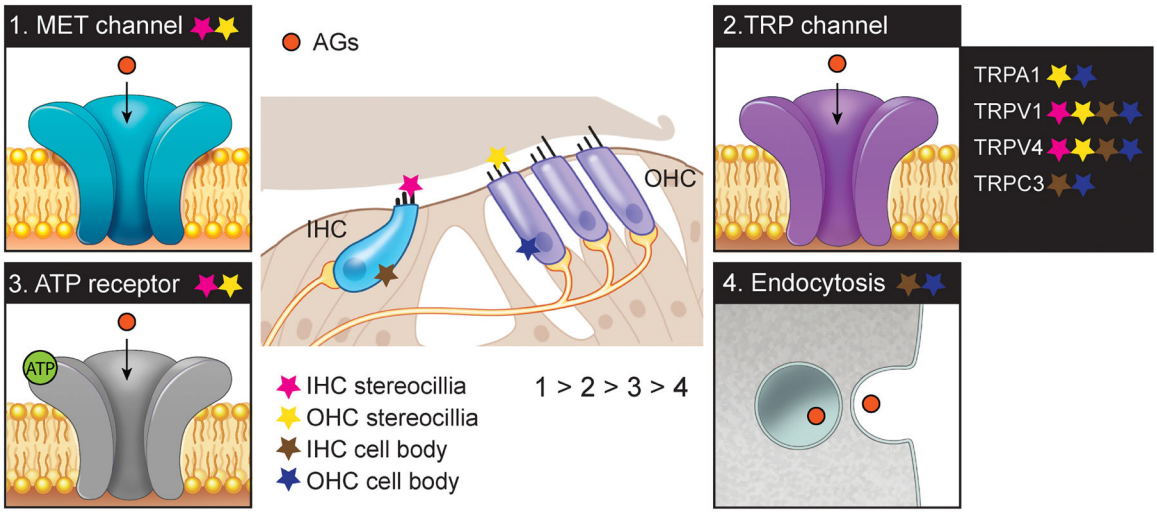

Fig. 4. Proposed mechanisms of aminoglycoside entry into hair cells.

Aminoglycoside (AG) enters into hair cells (IHCs and OHCs) through mechanotransducer (MET) (1), transient receptor potential (TRP) (2), adenosine triphosphate (ATP) receptor (3) channels, and endocytosis (4). Lower number depicts stronger evidence (1 > 2 > 3 > 4). Each star indicates the location of the channels or endocytosis in hair cells (pink, IHC stereocilia; yellow, OHC stereocilia; brown, IHC cell body; blue, OHC cell body).

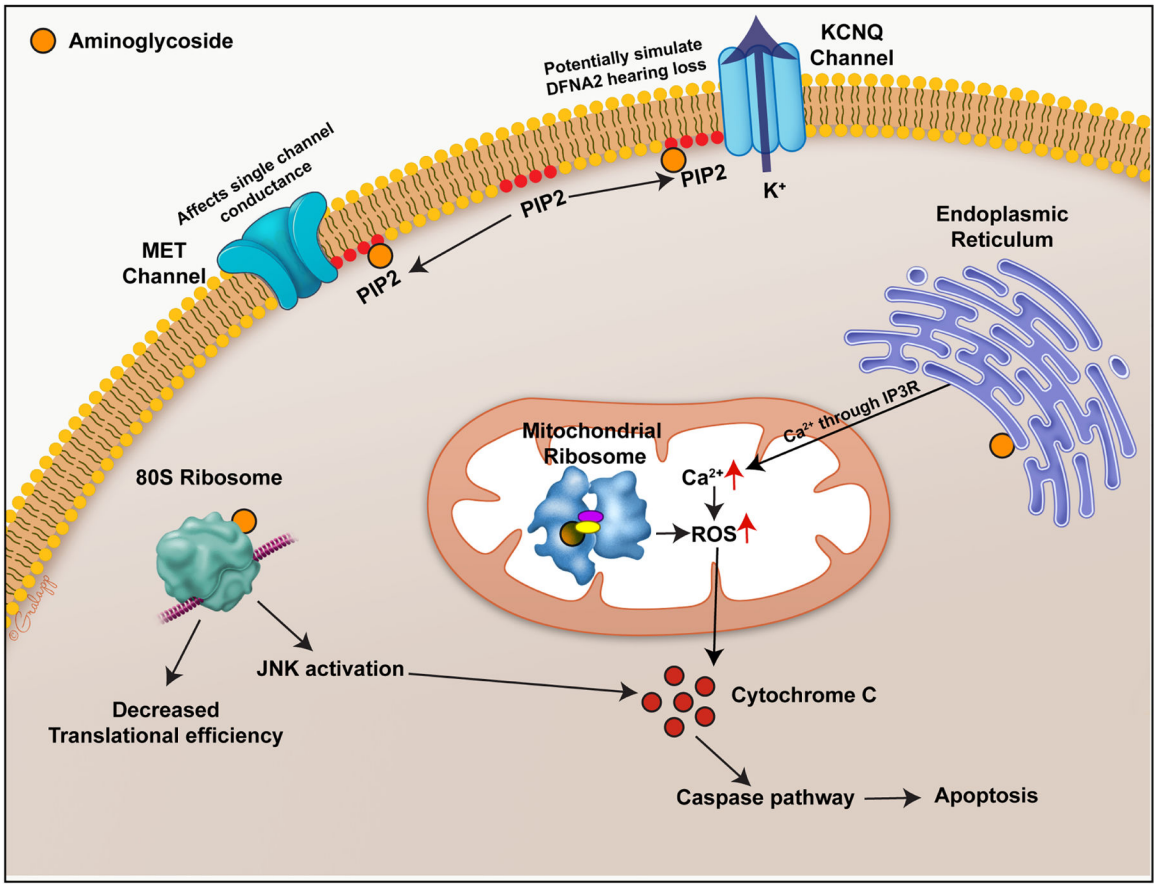

Fig. 5. Intracellular mechanism of aminoglycoside toxicity in hair cells.

A simplified schematic of the effect of aminoglycosides (AG) in hair cells. AG interacts with organelles like mitochondria, ribosomes and endoplasmic reticulum, triggering reactive oxygen species production. Excess of reactive oxygen species within the cells can trigger hair cell death. Interaction of AGs with the lipid bilayer components like PIP2 can render the hair cells nonfunctional.

Table 1:

Common ototoxic drugs

Drugs		References
Antibiotics		
	Neomycin	(Greenwood, 1959; Schacht, 1976a)
	Gentamicin	(Brummett et al., 1978)
	Tobramycin	(Brummett et al., 1978)
	Amikacin	(Brummett et al., 1978)
	Sisomicin	(Brummett et al., 1978)
	Kanamycin	(Sataloff et al., 1964)
	Spectinomycin	(Akiyoshi et al., 1976)
	Vancomycin	(Bailie and Neal, 1988; Cianfrone et al., 2011)
	Erythromycin	(Cianfrone et al., 2011)
	Azithromycin	(Cianfrone et al., 2011)
	Tetracycline	(Bisht and Bist, 2011)
Antimalarials		
	Quinine	(Jung et al., 1993; Tange et al., 1997)
	Chloroquine	(Davis et al., 2020; Hart and Naunton, 1964)
Chemotherapeutics		
	Cisplatin	(Fausti et al., 1984; Langer et al., 2013)
	Carboplatin	(Langer et al., 2013; Wake et al., 1994)
	Oxaliplatin	(Cassidy and Misset, 2002; Langer et al., 2013)
	Bleomycin	(Dal et al., 1973)
	Nitrogen mustard	(Asakuma et al., 1984; Cummings, 1968)
PDE5 inhibitors		
	Sildenafil	(Maddox et al., 2009; Mukherjee and Shivakumar, 2007)
	Tadalafil	(McGwin, 2010)
	Vardenafil	(Khan et al., 2011; Okuyucu et al., 2009)
Loop diuretics		
	Furosemide	(Brown et al., 1979; Cianfrone et al., 2011; Gallagher and Jones, 1979)
	Torsemide	(Cianfrone et al., 2011; Klinke and Mertens, 1988)
	Bumetanide	(Brown et al., 1979; Cianfrone et al., 2011)
NSAID		
	Salicylate	(Cazals, 2000; Jung et al., 1993)

Table 2:

List of aminoglycosides. Adapted and modified from (Wright et al., 1998)

Aminoglycoside	Source	Reference
Streptomycin	*Streptomyces griseus*	(Phaniendra et al., 2015; Schatz et al., 1944)
Spectinomycin	*Streptomyces spectabilis*	(Mason et al., 1961)
Neomycin	*Streptomyces fradiae*	(Waksman and Lechevalier, 1949)
Kanamycin	*Streptomyces kanamyceticlls*	(Umezawa et al., 1957)
Paromomycin	*Streptomyces rimasus NRRL 2234*	(TH Haskell, 1959)
Gentamicin	*Micromonospora purpurea*	(Weinstein, 1963)
Tobramycin	*Streptomyces tenebrarius*	(Higgins and Kastner, 1967)
Ribostamycin	*Streptomyces ribosidificus*	(Shomura et al., 1970)
Butirosin	*Bacillus circulans*	(Woo et al., 1971)
Sisomicin	*Micromonospora inyoesis*	(Weinstein et al., 1970)
Amikacin	Semisynthetic derivative of kanamycin B	(Kawaguchi et al., 1972)
Netilmicin	Semisynthetic derivative of sisomicin	(Kabins et al., 1976)
Isepamicin	Semisynthetic derivative of gentamicin B	(Nagabhushan et al., 1978)

Table 3:

Types of aminoglycosides. Adapted from (Wright et al., 1998).

2- Deoxystreptamine aminoglycosides		Other aminoglycosides
4,5- disubstituted	**4,6- disubstituted**	
Neomycin	Kanamycin	Hygromycin
Ribostamycin	Gentamicin	Streptomycin
Butirosin	Amikacin	Spectinomycin
Lividomycin	Netilmicin	Apramycin
Paromomycin	Tobramycin	Fortimicin
	Isepamicin	
	Sisomicin	
	Dibekacin	

Table 4.

Animal models for ototoxicity.

Species	AGs (dose)	Diuretics (dose)	Route	Reference
C	gentamicin (10, 20, 40, 125 mg/kg; single injection)	ethacrynic acid (40 mg/kg)	i.m.	(McFadden et al., 2002)
M	kanamycin (1000 mg/kg; single injection)	bumetanide (50 mg/kg)	i.p.	(Taylor et al., 2008)
M, R	kanamycin (400–900 mg/kg; 15d, twice daily)	-	s.c.	(Wu et al., 2001)
C	gentamicin (125 mg/kg; single injection)	ethacrynic acid (40 mg/kg)	i.m.	(Ding et al., 2010)
R	kanamycin (500 mg/kg; single injection)	ethacrynic acid (75 mg/kg)	i.m.	(Liu et al., 2011)
GP	gentamicin (100 mg/kg; single injection)	-	i.p.	(Imamura and Adams, 2003)
R	amikacin (200 mg/kg; 14, 28d, once daily)	-	i.m.	(El-Anwar et al., 2018)
GP	gentamicin (40, 80 mg/kg; 10d, once daily)	-	s.c.	(Bezdjian et al., 2015)
M	kanamycin (420, 550, 600 mg/kg; 3, 5, 7, 10, 14d, once daily)	furosemide (130 mg/kg)	s.c.	(Ju et al., 2017)
M	kanamycin (700, 1,000 mg/kg; single injection)	furosemide (100 mg/kg)	i.p.	(Jansen et al., 2013)
R	amikacin (200 mg/kg; 14d, once daily)	-	i.m.	(El-Anwar et al., 2016)
GP	amikacin (20, 400 mg/kg; 12, 30 days, once daily)	-	i.m.	(Oliveira et al., 2004)
M	kanamycin (900 mg/kg; 15d, twice daily)	furosemide (50 mg/kg)	i.p.	(Hirose and Sato, 2011)
G	gentamicin or kanamycin (4–500 mg/kg; single injection)	bumetanide (50 mg/kg) or furosemide (100 mg/kg)	s.c.	(Abbas and Rivolta, 2015)
GP	kanamycin (200 mg/kg; single injection)	furosemide (100 mg/kg)	s.c.	(Havenith et al., 2013)
GP	amikacin (200 mg/kg; 14 days, once daily)	-	s.c.	(Dirain et al., 2018)
R	amikacin (600 mg/kg; 14 days, once daily)	-	i.m.	(Aksoy et al., 2014)
GP, R	kanamycin (400 mg/kg; single injection)	ethacrynic acid (50 mg/kg)	s.c.	(Yamasoba et al., 2003)
R	tobramycin (160 mg/kg; 14 days, once daily)	-	s.c.	(Asplund et al., 2009)
M	kanamycin (1,000 mg/kg; single injection)	furosemide (400 mg/kg)	s.c.	(Schmitz et al., 2014)
M	kanamycin (1,000 mg/kg; single injection)	furosemide (500 mg/kg)	s.c.	(Fernandes and Lin, 2014)
R	kanamycin (500 mg/kg; single injection)	furosemide (200 mg/kg)	i.p.	(Chen et al., 2020)
M	kanamycin (700 mg/kg; 14d, twice daily)	-	s.c	(Koo et al., 2015)

(C, chinchilla; M, mouse; R, rat; GP, guinea pig; G, gerbil; i.m., intramuscular injection; i.p., intraperitoneal injection; s.c., subcutaneous injection)